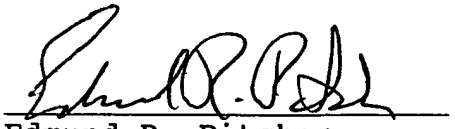
REMARKS

The foregoing amendments to the specification were approved in the parent of this Divisional Application. There are no outstanding issues of new matter as to any of the additions to the specification or claims. New claims 81 and 82 are simply a reformatting of the sequences claimed in claims 30 and 31 which have now been deleted. Support for these new claims can be found on page 62, for example, and in claims 30 and 31 as originally submitted. New claims 83-95 recite the novel amino acid sequences. Support for these claims can be found on pp. 63-65 of this specification, and in the claims as originally submitted.

Early favorable action on this application is respectfully requested.

Respectfully submitted,

LAHIVE & COCKFIELD



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December 4, 1990

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A series of consensus DNA sequences were designed with the goal of producing an active osteogenic protein. The sequences were based on partial amino acid sequence data obtained from the natural source product and on observed homologies with unrelated genes reported in the literature, or the sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in procaryotes, purified, cleaved, refolded, combined with a matrix, implanted in an established animal model, and shown to have endochondral bone-inducing activity. The currently preferred active totally biosynthetic proteins comprise two synthetic sequences designated COP5 and COP7. The amino acid sequences of these proteins are set forth below.

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          1        10          20          30          40
COP5           LYVDFS-DVGWDDWIVAPPGYQAFYCHGECPFPLAD
                    50          60          70
          HFNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
              80          90         100
          ISMLYLDENEKVVLKNYQEMVVEGCGCR

          1        10          20          30          40
COP7           LYVDFS-DVGWNDWIVAPPGYHAFYCHGECPFPLAD
                    50          60          70
          HLNSTN--H-AVVQTLVNSVNSKI--PKACCVPTELSA
              80          90         100
          ISMLYLDENEKVVLKNYQEMVVEGCGCR
```

In these sequences and all other amino acid sequences disclosed herein, the dashes (-) are used as fillers only to line up comparable sequences in related proteins, and have no other function. Thus, amino acids 46-50 of COP7, for example, are NHAVV. Also, the numbering of amino acids is selected solely

molecular weight of about 27 kD. When reduced, the 27 kD protein gives rise to the two deglycosylated polypeptides have molecular weights of about 14 kD to 16 kD.

Analysis of digestion fragments indicate that the native 30 kD osteogenic protein contains the following amino acid sequences (question marks indicate undetermined residues):

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;
(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;
(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;
(4) M-S-S-L-S-I-L-F-F-D-E-N-K;
(5) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;
(6) V-D-F-A-D-I-G;
(7) V-P-K-P-C-C-A-P-T;
(8) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;
(9) D-I-G-?-S-E-W-I-I-?-P;
(10) S-I-V-R-A-V-G-V-V-P-G-I-P-E-P-?-?-V;
(11) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;
(12) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;
(13) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;
(14) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-E-P-R-N-?-A-R-R-Y-L;
(15) A-R-R-K-Q-W-I-E-P-R-N-?-A-?-R-Y-?-?-V-D; and
(16) R-?-Q-W-I-E-P-?-N-?-A-?-?-Y-L-K-V-D-?-A-?-?-G.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1A represents the nucleotide sequence of the genomic copy of osteogenic protein "OP1" gene. The unknown region between 1880 and 1920 actually represents about 1000 nucleotides;

FIGURE 1B is a representation of the hybridization of the consensus gene/probe to the osteogenic protein "OP1" gene;

FIGURE 2(A-D) is a collection of plots of protein concentration (as indicated by optical absorption) vs elution volume illustrating the results of bovine osteogenic protein (BOP) fractionation during purification on (A) heparin-Sepharose I; (B) HAP-Ultragel; (C) sieving gel (Sephacryl 300); and (D) heparin-Sepharose II;

FIGURE 3 is a photographic reproduction of a Coomassie blue stained SDS polyacrylamide gel of the osteogenic protein under non-reducing (A) and reducing (B) conditions;

FIGURE 4 is a photographic reproduction of a Con A blot of an SDS polyacrylamide gel showing the carbohydrate component of oxidized (A) and reduced (B) 30 kD protein;

FIGURE 5 is a photographic reproduction of an autoradiogram of an SDS polyacrylamide gel of ^{125}I-labelled glycosylated (A) and deglycosylated (B) osteogenic protein under non-reducing (1) and reducing (2) conditions;

FIGURE 6 is a photographic reproduction of an autoradiogram of an SDS polyacrylamide gel of peptides produced upon the digestion of the 30 kD osteogenic protein with V-8 protease (B), Endo Lys C protease (C), pepsin (D), and trypsin (E). (A) is control;

FIGURE 7(A-C) is a collection of HPLC chromatograms of tryptic peptide digestions of 30 kD BOP (A), the 16 kD subunit (B), and the 18 kD subunit (C);

FIGURE 8 is an HPLC chromatogram of an elution profile on reverse phase C-18 HPLC of the samples recovered from the second heparin-Sepharose chromatography step (see FIGURE 2D). Superimposed is the percent bone formation in each fraction;

FIGURE 9 is a gel permeation chromatogram of an elution profile on TSK 3000/2000 gel of the C-18 purified osteogenic peak fraction. Superimposed is the percent bone formation in each fraction;

FIGURE 10(A-D) is a collection of graphs of protein concentration (as indicated by optical absorption) vs. elution volume illustrating the results of human protein fractionation on

heparin-Sepharose I (A), HAP-Ultragel (B), TSK 3000/2000 (C), and heparin-Sepharose II (D). Arrows indicate buffer changes;

FIGURE 11 is a graph showing five representative dose response curves, labelled A-E, for bone-inducing activity in samples taken from the different column purification steps: reverse phase HPLC on C-18 (A), Heparin-Sepharose II (B), Sephacryl S-300 HR (C), HAP-ultragel (D), and Heparin-Sepharose I (E);

FIGURE 12 is a bar graph of radiomorphometric analyses of feline bone defect repair after treatment with an osteogenic device (A), carrier control (B), and demineralized bone (C);

FIGURE 13 is a schematic representation of the DNA sequence and corresponding amino acid sequence of a consensus gene/probe for osteogenic protein (COPO);

FIGURE 14 is a graph of osteogenic activity, measured by calcium content vs. increasing molecular weight, showing peak bone forming activity in the 30 kD region of an SDS polyacrylamide gel;

FIGURE 15 is a photographic representation of a Coomassie blue stained SDS gel showing gel purified subunits of the 30 kD protein;

FIGURE 16 is a pair of HPLC chromatograms of Endo Asp N proteinase digests of the 18 kD subunit (A) and the 16 kD subunit (B);

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FIGURE 17 is a photographic representation of the histological examination of bone implants in the rat model: carrier alone (A); carrier and glycosylated osteogenic protein (B); and carrier and deglycosylated osteogenic protein (C). Arrows indicate osteoblasts;

FIGURE 18 is a comparison of the amino acid sequence of various osteogenic proteins to those of the TGF-beta family. COP1, COP3, COP4, COP5, and COP7 are a family of analogs of synthetic osteogenic proteins developed from the consensus gene that was joined to a leader protein via a hinge region having the sequence D-P-N-G that permitted chemical cleavage at the D-P site (by acid) or N-G (by hydroxylamine) resulting in the release of the analog protein; VGI is a Xenopus protein, DPP is a Drosophila protein; OP1 is a native osteogenic protein; CBMP2a and 2b, and CBMP3 are subparts of proteins disclosed in PCT application 087/01537; MIS is Mullerian inhibitory substance; and "consensus choices" represent various substitutions of amino acids that may be made at various positions in osteogenic proteins;

FIGURE 19 is a graph showing the specific activity, as measured by alkaline phosphatase activity, of osteogenic devices comprising heparin-Sepharose II-purified naturally sourced OP and allogenic (rat) bone matrix, or xenogenic (bovine and deglycosylated bovine) bone matrix;

FIGURES 20A and 20B are bar graphs showing the specific activity of naturally sourced OP before, gel elution (A), and after gel elution (B), as measured by calcium content vs. increasing concentrations of proteins (dose curve, in ng);.

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B5(b)Protein Estimation Using Gel Scanning Techniques

A standard curve is developed employing known amounts of a standard protein, bovine serum albumin. The protein at varying concentration (50-300 ng) is loaded on 15% SDS gel, electrophoresed, stained in comassie and destained. The gel containing standard proteins is scanned at predetermined settings using a gel scanner at 580 nm. The area covered by the protein band is calculated and a standard curve against concentrations of protein is constructed. A sample with an unknown protein concentration is electrophoresed with known concentration of BSA. The lane contained unknown sample is scanned and from the area the concentration of protein is determined.

B5(c)Gel Elution and Specific Activity

An aliquot of C-18 highly purified active fraction is subjected to SDS gel and sliced according to molecular weights described in Figure 14. Proteins are eluted from the slices in 4 M guanidine-HCl containing 0.5% Triton X-100, desalted, concentrated and assayed for endochondral bone forming activity as determined by calcium content. The C-18 highly active fractions and gel eluted substantially pure 30 kD osteogenic protein are implanted in varying concentrations in order to determine the half maximal bone inducing activity.

Figure 14 demonstrates that the bone inducing activity is due to proteins eluted at 28-34 kD region. The recovery of activity after the gel elution step is determined by calcium content.

species at about 27 kD (FIGURE 5B-1). Upon reduction, the 27 kD species is reduced to species having a molecular weight of about 14 kD - 16 kD (FIGURE 5B-2).

Chemical cleavage of the carbohydrate moieties using hydrogen fluoride (HF) is performed to assess the role of carbohydrate on the bone inducing activity of BOP in vivo. Active osteogenic protein fractions pooled from the C-18 chromatography step are dried in vacuo over P_2O_5 in a polypropylene tube, and 50 ml freshly distilled anhydrous HF at -70°C is added. After capping the tube tightly, the mixture is kept at 0°C in an ice-bath with occasional agitation for 1 hr. The HF is then evaporated using a continuous stream of dry nitrogen gas. The tube is removed from the ice bath and the residue dried in vacuo over P_2O_5 and KOH pellets.

Following drying, the samples are dissolved in 100 ml of 50% acetonitrile/0.1% TFA and aliquoted for SDS gel analysis, Con A binding, and biological assay. Aliquots are dried and dissolved in either SDS gel sample buffer in preparation for SDS gel analysis and Con A blotting or 4 M guanidine-HCl, 50mM Tris-HCl, pH 7.0 for biological assay.

The results show that samples are completely deglycosylated by the HF treatment: Con A blots after SDS gel electrophoreses and transfer to Immobilon membrane showed no binding of Con A to the treated samples, while untreated controls were strongly positive at 30 kD. Coomassie gels of treated samples showed the presense of a 27 kD band instead of the 30 kD band present in the untreated controls.

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The 16 kD and 18 kD subunits are digested with Endo Asp N proteinase. The protein is treated with 0.5 mg EndoAsp-N in 50mM sodium phosphate buffer, pH 7.8 at 36°C for 20 hr. The conditions for fractionation are the same as those described previously for the 30 kD, 16 kD, and 18 kD digests. The profiles obtained are shown in FIGUREs 16A and 16B.

Various of the peptide fragments produced using the foregoing procedures have been analyzed in an automated amino acid sequencer (Applied Biosystems 470A with 120A on-line PTH analysis). The following sequence data has been obtained:

(1) S-F-D-A-Y-Y-C-S-G-A-C-Q-F-P-M-P-K;
(2) S-L-K-P-S-N-Y-A-T-I-Q-S-I-V;
(3) A-C-C-V-P-T-E-L-S-A-I-S-M-L-Y-L-D-E-N-E-K;
(4) M-S-S-L-S-I-L-F-F-D-E-N-K;
(5) V-G-V-V-P-G-I-P-E-P-C-C-V-P-E;
(6) V-D-F-A-D-I-G;
(7) V-P-K-P-C-C-A-P-T;
(8) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-?-P;
(9) D-I-G-?-S-E-W-I-I-?-P;
(10) S-I-V-R-A-V-G-V-V-P-G-I-P-E-P-?-?-V;
(11) D-?-I-V-A-P-P-Q-Y-H-A-F-Y;
(12) D-E-N-K-N-V-V-L-K-V-Y-P-N-M-T-V-E;
(13) S-Q-T-L-Q-F-D-E-Q-T-L-K-?-A-R-?-K-Q;

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(14) D-E-Q-T-L-K-K-A-R-R-K-Q-W-I-E-P-R-N-?-A-R-R-Y-L;

(15) A-R-R-K-Q-W-I-E-P-R-N-?-A-?-R-Y-?-?-V-D; and

(16) R-?-Q-W-I-E-P-?-N-?-A-?-?-Y-L-K-V-D-?-A-?-?-G.

C6. Amino Acid Analysis

Strategies for obtaining amino acid composition were developed using gel elution from 15% SDS gels, transfer onto Immobilon, and hydrolysis. Immobilon membrane is a polymer of vinylidene difluoride and, therefore, is not susceptible to acid cleavage. Samples of oxidized (30 kD) and reduced (16 kD and 18 kD) BOP are electrophoresed on a gel and transferred to Immobilon for hydrolysis and analysis as described below. The composition data generated by amino acid analyses of 30 kD BOP is reproducible, with some variation in the number of residues for a few amino acids, especially cysteine and isoleucine.

Samples are run on 15% SDS gels, transferred to Immobilon, and stained with Coomassie blue. The bands of interest are excised from the Immobilon, with a razor blade and placed in a 6 x 50mm Corning test tube cleaned by pyrolysis at 550°C. When cysteine is to be determined, the samples are treated with performic acid, which converts cysteine to cysteic acid. Cysteic acid is stable during hydrolysis with HCl, and can be detected during the HPLC analysis by using a modification of the normal Pico-Tag eluents (Millipore) and gradient. The performic acid is made by mixing 50 ml 30% hydrogen

salt; the bound proteins are eluted by 0.5 M NaCl (FIGURE 10D. FIGURE 10C describes the elution profile for the intervening gel filtration step described above). Assaying the heparin-Sepharose fractions shows that the bound fraction eluted by 0.5M NaCl have bone-inducing activity. The active fraction is then subjected to C-18 reverse phase chromatography.

The active fraction can then be subjected to SDS-PAGE as noted above to yield a band at about 30 kD comprising substantially pure human osteogenic protein.

E. BIOSYNTHETIC PROBES FOR ISOLATION OF GENES ENCODING NATIVE OSTEOGENIC PROTEIN

E-1 PROBE DESIGN

A synthetic consensus gene shown in FIGURE 13 was designed as a hybridization probe (and to encode a consensus protein, see below) based on amino acid predictions from homology with the TGF-beta gene family and using human codon bias as found in human TGF-beta. The designed concensus sequence was then constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer.

Tryptic peptides derived from BOP and sequenced by Edman degradation provided amino acid sequences that showed strong homology with the Drosophila DPP protein sequence (as inferred from the gene), the Xenopus VG1 protein, and somewhat less homology to inhibin and TGF-beta, as demonstrated below in TABLE 6.

E. Production of Antisera

Antisera to COP 7 and COP5 were produced in New Zealand white rabbits. Western blots demonstrate that the antisera react with COP 7 and COP5 preparations. Antisera to COP 7 has been tested for reactivity to bovine osteogenic protein samples. Western blots show a clear reaction with the 30kD protein and, when reduced, with the 16kD subunit. The immunoreactive species appears as a closely-spaced doublet in the 16K subunit region, similar to the 16K doublet seen in Con A blots.

III. MATRIX PREPARATION

A. General Consideration of Matrix Properties

The carrier described in the bioassay section, infra, may be replaced by either a biodegradable-synthetic or synthetic-inorganic matrix (e.g., HAP, collagen, tricalcium phosphate, or polylactic acid, polyglycolic acid and various copolymers thereof). Also xenogeneic bone may be used if pretreated as described below.

Studies have shown that surface charge, particle size, the presence of mineral, and the methodology for combining matrix and osteogenic protein all play a role in achieving successful bone induction. Perturbation of the charge by chemical modification abolishes the inductive response. Particle size influences the quantitative response of new bone; particles between 75 and 420 mm elicit

useful for quantitation and obtaining an estimate of bone formation very quickly after the implants are removed from the rat. In order to estimate the amount of bone formation, the calcium content of the implant is determined.

Implants containing osteogenic protein at several levels of purity have been tested to determine the most effective dose/purity level, in order to seek a formulation which could be produced on an industrial scale. The results are measured by specific acivity of alkaline phosphatase and calcium content, and histological examination. The specific activity of alkaline phosphatase is elevated during onset of bone formation and then declines. On the other hand, calcium content is directly proportional to the total amount of bone that is formed. The osteogenic activity due to osteogenic protein is represented by "bone forming units". For example, one bone forming unit represents the amount of protein that is needed for half maximal bone forming activity as compared to rat demineralized bone matrix as control and determined by calcium content of the implant on day 12.

E. Results

E-1. Natural Sourced Osteogenic Protein

Dose curves are constructed for bone inducing activity in vivo at each step of the purification scheme by assaying various concentrations of protein. FIGURE 11 shows representative dose curves in rats as determined by

alkaline phosphatase. Similar results are obtained when represented as bone forming units. Approximately 10-12 mg of the Sephacryl-fraction, 3-4 mg of heparin-Sepharose-II fraction, 0.4-0.5 mg of the C-18 column purified fraction, and 20-25 ng of gel eluted highly purified 30 kD protein is needed for unequivocal bone formation (half maximum activity). 20-25 ng per 25 mg of implant is normally sufficient to produce endochondral bone. Thus, 1-2 ng osteogenic protein per mg of implant is a reasonable dosage, although higher dosages may be used. (See section IB5 on specific activity of osteogenic protein.)

E-2. Xenogenic Matrix Results

Deglycosylated xenogenic collagenous bone matrix (example: bovine) has been used instead of allogenic collagenous matrix to prepare osteogenic devices (see previous section) and bioassayed in rat for bone inducing activity in vivo. The results demonstrate that xenogenic collagenous bone matrix after chemical deglycosylation induces successful endochondral bone formation (Figure 19). As shown by specific activity of alkaline phosphatase, it is evident that the deglycosylated xenogenic matrix induced bone whereas untreated bovine matrix did not.

Histological evaluation of implants suggests that the deglycosylated bovine matrix not only has induced bone in a way comparable to the rat residue matrix but also has advanced the developmental stages that are involved in endochondral bone differentiation. Compared to rat residue as control,

the HF treated bovine matrix contains extensively remodeled bone. Ossicles are formed that are already filled with bone marrow elements by 12 days. This profound action as elicited by deglycosylated bovine matrix in supporting bone induction is reproducible and is dose dependent with varying concentration of osteogenic protein.

E-3. Synthetic/Recombinant Proteins (COP5, COP7)

The device that contained only rat carrier showed complete absence of new bone formation. The implant consists of carrier rat matrix and surrounding mesenchymal cells. Again, the devices that contained rat carrier and not correctly folded (or biologically inactive) recombinant protein also showed complete absence of bone formation. These implants are scored as cartilage formation (-) and bone formation (-). The endochondral bone formation activity is scored as zero percent (0%). (FIGURE 22A)

Implants that included biologically active recombinant protein, however, showed evidence of endochondral bone formation. Histologically they showed new cartilage and bone formation.

The cartilage formation is scored as (+) by the presence of metachromatically stained chondrocytes in center of the implant, as (++) by the presence of numerous chondrocytes in many areas of the implant and as (+++) by the presence of abundant chondrocytes forming cartilage matrix and the appearance of hypertrophied chondrocytes accompanying cartilage calcification (FIGURE 22B).

A-1. Procedure

Sixteen adult cats weighing less than 10 lbs. undergo unilateral preparation of a 1 cm bone defect in the right femur through a lateral surgical approach. In other experiments, a 2 cm bone defect was created. The femur is immediately internally fixed by lateral placement of an 8-hole plate to preserve the exact dimensions of the defect. There are three different types of materials implanted in the surgically created cat femoral defects: group I (n = 3) is a control group which undergo the same plate fixation with implants of 4 M guanidine-HCl-treated (inactivated) cat demineralized bone matrix powder (GuHCl-DBM) (360 mg); group II (n = 3) is a positive control group implanted with biologically active demineralized bone matrix powder (DBM) (360 mg); and group III (n = 10) undergo a procedure identical to groups I-II, with the addition of osteogenic protein onto each of the GuHCl-DBM carrier samples. To summarize, the group III osteogenic protein-treated animals are implanted with exactly the same material as the group I animals, but with the singular addition of osteogenic protein.

All animals are allowed to ambulate ad libitum within their cages post-operatively. All cats are injected with tetracycline (25 mg/kg SQ each week for four weeks) for bone labelling. All but four group III animals are sacrificed four months after femoral osteotomy.